(12) UK Patent Application (19) GB (11) 2 218 101 A

(21) Application No 8907148.4

(22) Date of filing 30.03.1989

(30) Priority data
(31) 8807803   (32) 31.03.1988   (33) GB

(71) Applicant
Glaxo Group Limited

(Incorporated in the United Kingdom)

Clarges House, 6-12 Clarges Street, London,
W1Y 8DH, United Kingdom

(72) Inventors
Jean-Michel Dayer
Phillppe Lucien Seckinger

(74) Agent and/or Address for Service
C L Brewer
Glaxo Holdings Limited, 63 Graham Street, London,
N1 8JZ, United Kingdom

(51) INT CL⁴
C07K 3/28

(52) UK CL (Edition J)
C3H HFZ H100 H106 H107 H109 H115 H140
U1S S1328

(56) Documents cited
None

(58) Field of search
UK CL (Edition J) C3H HFX HFZ HF1 HF2AA
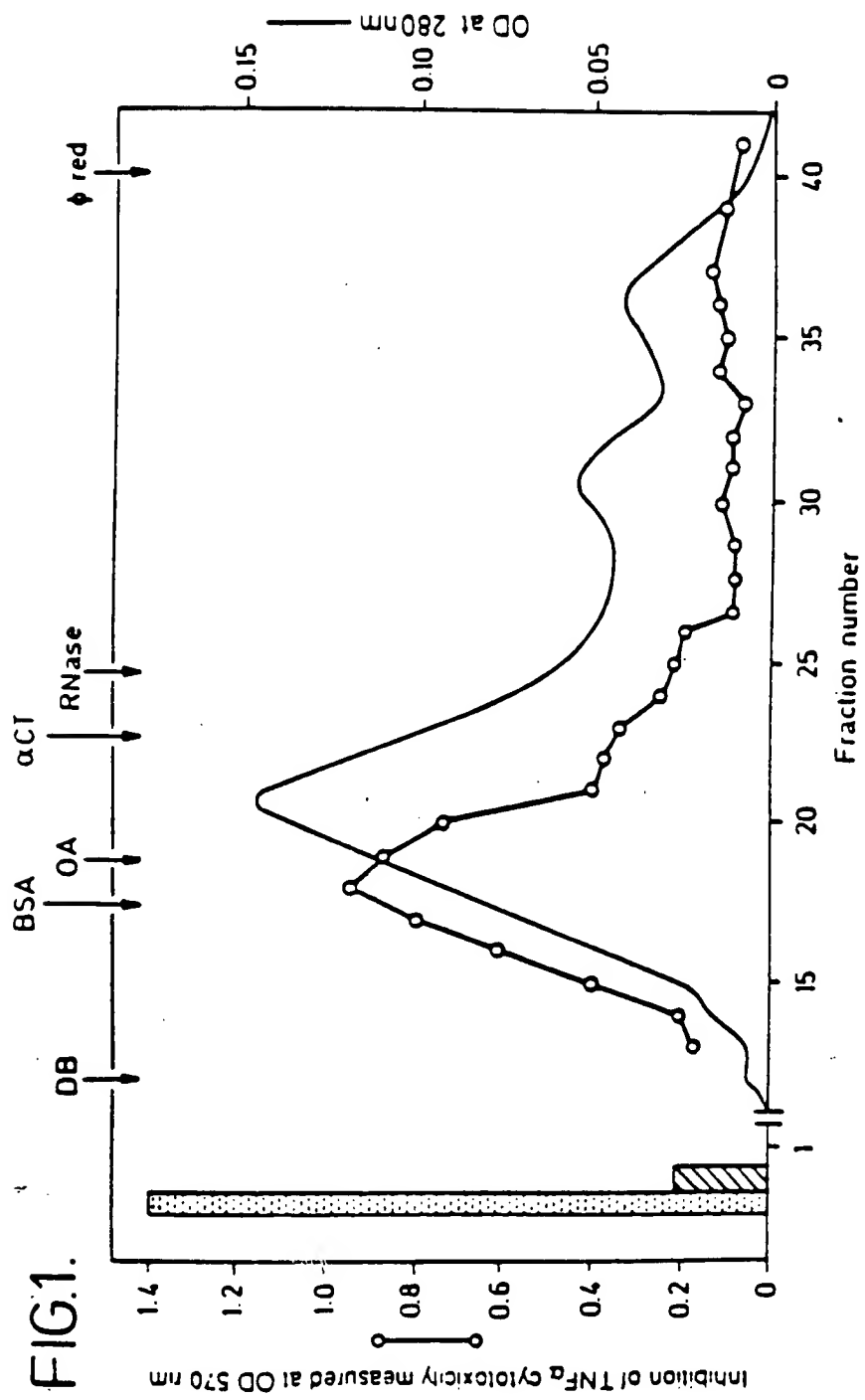HF2AB HF2AC HF2AX HF3 HF4 HF5
INT CL⁴ C07K
DIALOG ONLINE COMPUTER SEARCH (BIOTECH.
WPI).

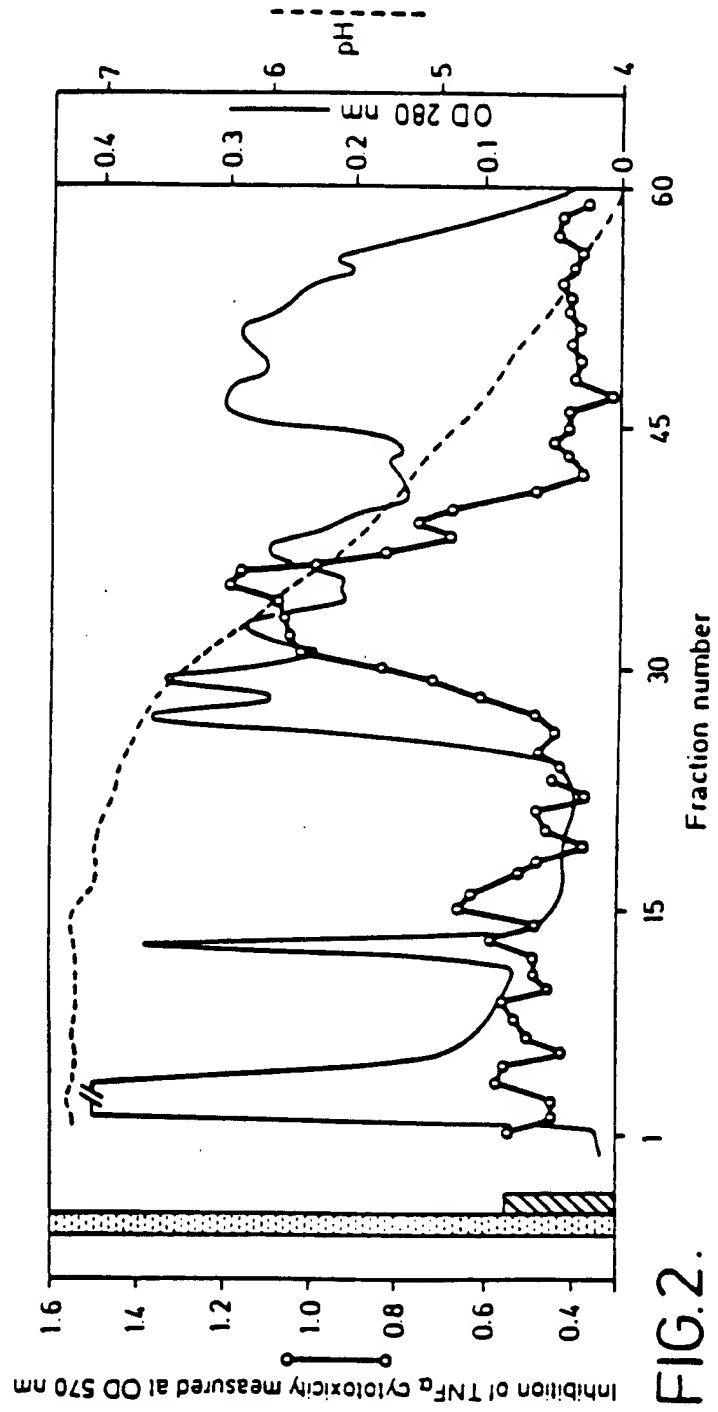(54) TNF-alpha Inhibitors

(57) A protein having a selective tumour necrosis factor (TNF)-α inhibitory activity, but which does not block other proteins, particularly IL-1, is prepared by extraction from urine of febule patients.
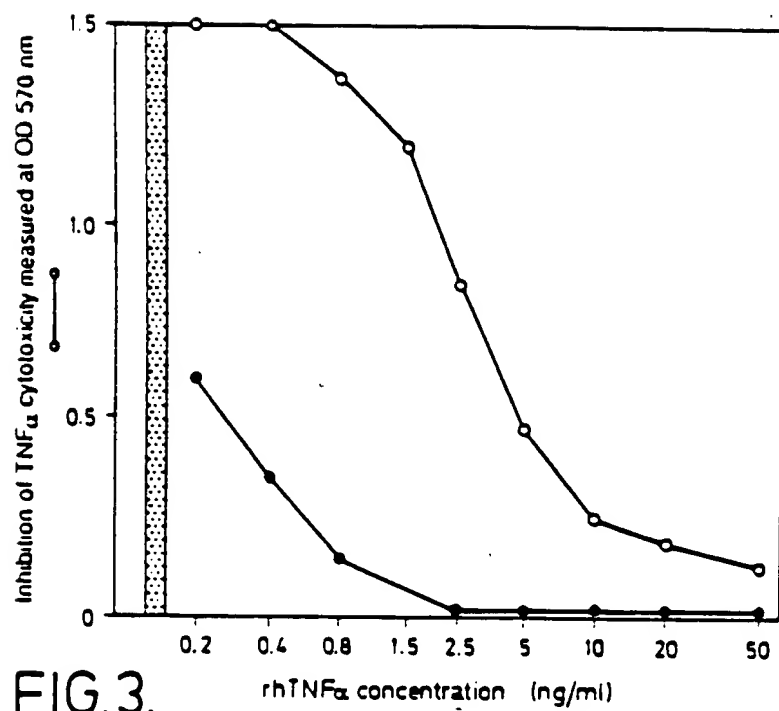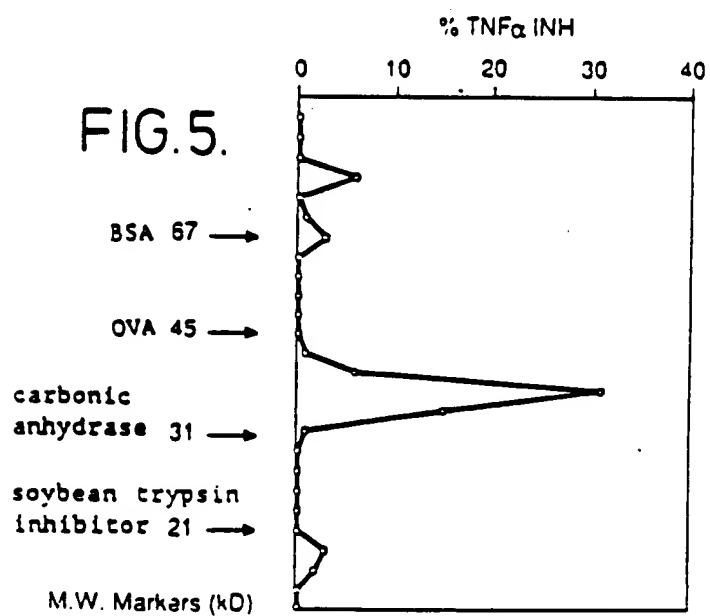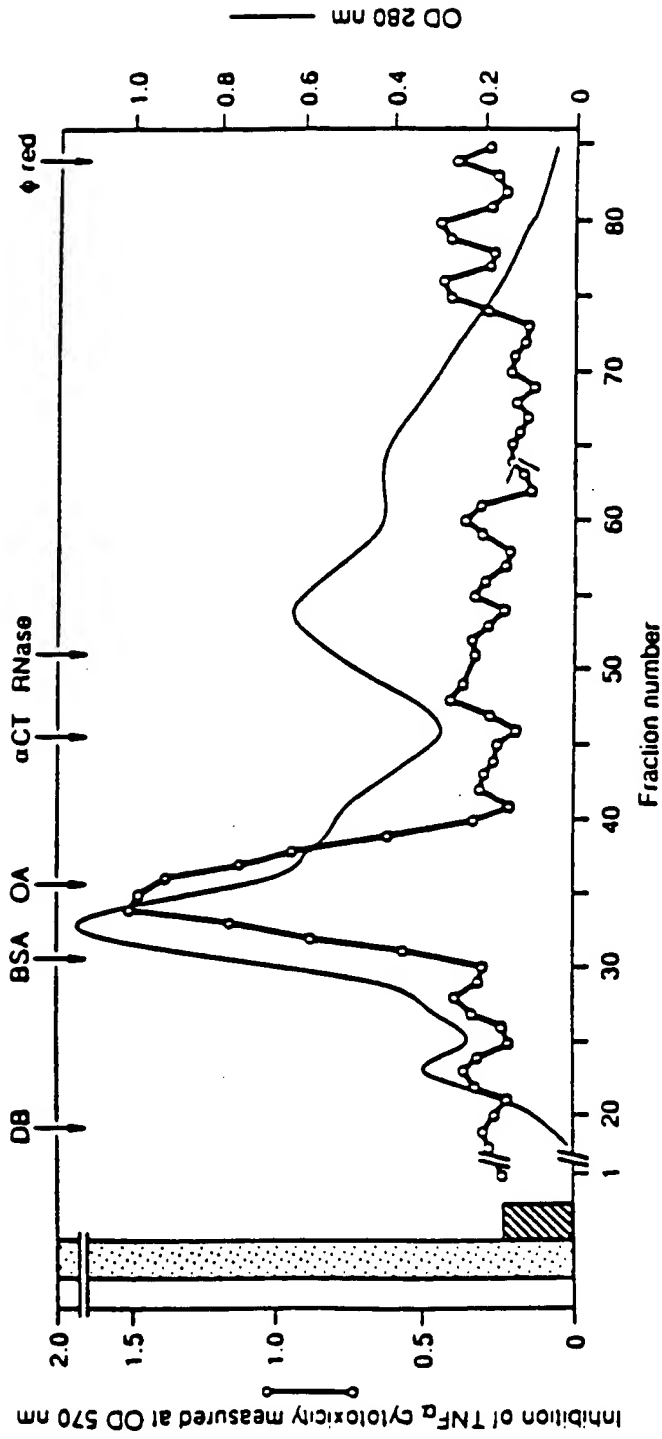
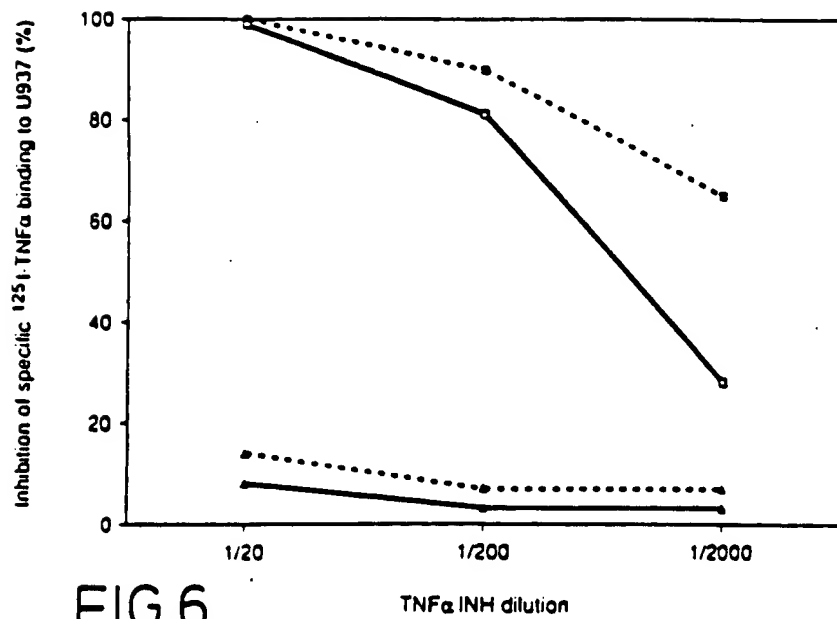GB 2 218 101 A

FIG1.

FIG.2.

FIG.3.

FIG.5.

FIG.4.

FIG.6.

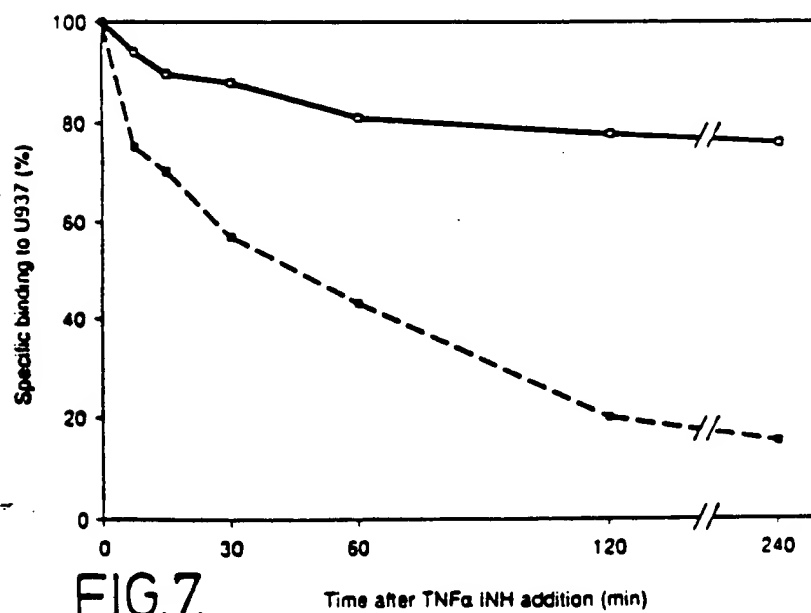
FIG.7.

BIOLOGICALLY ACTIVE PROTEINS

The present invention relates to a novel protein having an inhibitory effect against Tumour Necrosis Factor α-mediated activity, to the isolation and purification of such a protein from natural sources, to its preparation by DNA manipulation and to the use of such a protein in the treatment of conditions associated with excessive or unregulated TNFα production.

Tumour necrosis factor (TNF) is an activity embodied by a family of at least two proteins, α and β, which are cytotoxic for tumour cells and inhibit their growth in culture [E. Carswell et. al. "An endotoxin-induced serum factor that causes necrosis of tumours", Proc. Natl. Acad. Sci. USA, 72, p3666 (1975)]. Tumour necrosis factor α (TNFα), also termed "cachectin", is mainly produced by cells of the monocyte/macrophage lineage in response to "stress" signals which accompany invasive stimuli such as bacteria, viruses, tumours and other toxins. TNFβ, commonly termed "lymphotoxin", is mainly produced by lymphoid cells. TNFβ has many activities similar to those of TNFα, but it appears to be less potent although this may be as a result of difficulties in preparing pure TNFβ.

TNFα mediates and participates in a wide range of biological activities [B. Beutler et. al., "Identity of tumour necrosis factor and the macrophage-secreted factor cachectin", Nature, 316, p552 (1985)] sharing several of them with interleukin 1 (IL-1) [J. Le et. al., "Tumour necrosis factor and interleukin 1 : cytokines with multiple overlapping biological activities", Laboratory Invest., 56, p234 (1987)]. Elevated levels of TNFα induced by, for example, tumour

cells may lead to weight loss and cachexia and TNFα has also been implicated as a principal mediator of endotoxic shock (septic shock) which can be fatal. Other biological effects of TNFα include hypotension, fever (induced by stimulation of hypothalamic prostaglandin E_2 (PGE_2) synthesis), coagulopathy (induced by stimulation of vascular endothelial cells which release, for example, tissue factor) and tissue destruction (induced by, for example, stimulation of a series of proteinases, including collagenase production by dermal fibroblasts and synovial cells) [C. Dinarello et. al., "Tumour necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1", J. Exp. Med., 163, p1433 (1986); J. Dayer et. al., "Cachectin/tumour necrosis factor stimulate collagenase and prostaglandin E_2 production by human dermal fibroblasts and synovial cells", J. Exp. Med., 162, p2163 (1985)].

There exists, therefore, a need to develop a cachectin/TNFα inhibitor which prevents endotoxic shock, cachexia and the other deleterious effects described above. It has been shown that passive immunisation of animals against cachectin can prevent endotoxin-induced death, mediated by TNFα antibodies [B. Beutler et. al., Nature, 316, supra].

We have now identified a novel protein which has a potent inhibitory effect against TNFα-mediated activities without significant concomitant inhibition of IL-1-mediated activity. The protein is hereinafter identified as Tumour Necrosis Factor α Inhibitor (TNFα INH).

Thus in one aspect of the invention, we provide a protein which selectively inhibits tumour necrosis factor α-mediated activity.

As used herein, selective inhibition as shown by the inhibitor of the invention is identified as the ability to block TNF-mediated activity while lacking the ability to block other proteins which have in common with TNF certain but not all of the biological activities of TNF, such as IL-1.

Preferably, the tumour necrosis factor α inhibitor of the invention is in a substantially homogeneous form, being substantially free from major contaminents and/or substantially free from other proteinaceous material.

The tumour necrosis factor α inhibitor according to the invention has been found to have one or more of the following characteristics:

(a) a molecular weight in the range 40 to 60 kDa, determined by molecular sieve chromatography;

(b) an iso-electric point (pI) in the range 5.5 to 6.1, determined by chromatofocussing;

(c) inhibition of the standard TNF assay of differential cytotoxicity for murine L929 cells treated with actinomycin D, as described by G. Nedwin et. al. "Effects of interleukin 2, interferon-γ and mitogens on the production of tumour necrosis factors α and β", J. Immunol., 135, p2492 (1985). This inhibition can be overcome by further addition of TNFα, indicating that the inhibition is competitive. The inhibitor is also an inhibitor of TNFβ activity, although inhibition of TNFα in this assay is more efficient than that of TNFβ;

(d) inhibition of TNF-induced PGE_2 release from human fibroblasts and synovial cells;

(e) the inhibitor interferes with the binding of TNFα to U937 cells (a monocytic tumour line) as evidenced by inhibition of binding of radiolabelled TNFα (^{125}I-TNFα);

(f) the dissociation of a pre-formed TNFα : U937 cell complex is promoted by the inhibitor in a temperature dependent manner;

(g) the inhibitor does not degrade TNF by proteolytic cleavage;

(h) the inhibitor does not inhibit IL-1 receptor-binding activity e.g. the binding of radiolabelled IL-1 (^{125}I-IL-1α) to the murine thymoma subline EL4-6.1.

We have found that the protein of the invention when further purified has a molecular weight of about 33000 daltons as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

There is thus provided as a further or alternative aspect, a protein which selectively inhibits TNFα-mediated activity which has one or more of the following characteristics :

(a) a molecular weight of about 33 kDa, determined by SDS PAGE;

(b) an iso-electric point (pI) in the range 5.5 to 6.1, determined by chromatofocussing;

(c) inhibition of the standard TNF assay of differential cytotoxicity for murine L929 cells treated with actinomycin D, as described by G. Nedwin et. al. "Effects of interleukin 2, interferon-γ and mitogens on the production of tumour necrosis factors α and β", J. Immunol., 135, p2492 (1985). This inhibition can be overcome by further addition of TNFα, indicating that the inhibition is

competitive. The inhibitor is also an inhibitor of TNFβ activity,
although inhibition of TNFα in this assay is more efficient than that
of TNFβ.;

(d) inhibition of TNF-induced PGE release from human
fibroblasts and synovial cells;

(e) the inhibitor interferes with the binding of TNFα to U937
cells (a monocytic tumour line) as evidenced by inhibition of binding
of radiolabelled TNFα (^{125}I-TNFα);

(f) the dissociation of a pre-formed TNFα : U937 cell complex
is promoted by the inhibitor in a temperature dependent manner;

(g) the inhibitor does not degrade TNF by proteolytic
cleavage;

(h) the inhibitor does not inhibit IL-1 receptor-binding
activity e.g. the binding of radiolabelled IL-1 (^{125}I-IL-1α) to the
murine thymoma subline EL4-6.1.

Preferably the TNFα INH of the present invention has both of the
characteristics (a) and (b) and one or more of the characteristics (c)
to (h).

In particular, the TNFα INH of the present invention has all of
the characteristics (a) to (h).

The protein of the invention has an amino terminal amino acid
sequence as follows :

Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-

Pro-Gln-Cys-Asn-Ser-Ile

It is further believed that the next three amino acids provide a
glycosylation site and that the sequence thus continues
Asn-Ser-Thr-Lys.

It will be appreciated that a TNFα inhibitor according to the invention will comprise an amino acid sequence substantially corresponding to the sequence of native TNFα INH and containing an amino terminal sequence substantially identical to that described above. The sequence of a TNFα inhibitor according to the invention will thus be identical to the sequence of native TNFα INH or contain one or more deletions, substitutions, insertions, inversions or additions of allelic origin or otherwise, the resulting sequence will have at least 80% and preferably 90%, homology with the sequence of native TNFα INH and retain essentially the same biological properties of the protein.

The TNFα inhibitor of the invention has been demonstrated to be proteinaceous in that it is inactivated by heating in a time and temperature dependent manner is is destroyed by treatment with trypsin or pronase.

The TNFα INH of the invention has also been shown to be a glycoprotein since treatment with the enzyme Endoglycosidase F reduces the molecular weight by 7 to 8 kDa.

In a further or alternative aspect of the invention there is thus provided a TNFα inhibitor as defined herein, but which is in a substantially unglycosylated state.

The inhibitors of the invention are of interest in the treatment of conditions in which it is desirable to inhibit TNFα activity, for example, those which arise from the effects of TNFα such as weight loss, shock, cachexia and chronic local inflammation, rheumatoid arthritis, disseminated intravascular coagulation and nephritis.

There is thus provided as a further aspect of the invention a TNFα inhibitor as herein defined or a pharmaceutically acceptable derivative thereof for use as an active therapeutic agent, in particular, in the treatment of conditions associated with excessive or unregulated TNFα production.

In a further or alternative aspect of the invention there is provided a method for the treatment of conditions associated with excessive or unregulated TNFα production in a mammal including man comprising administration of an effective amount of a TNFα inhibitor as herein defined or a pharmaceutically acceptable derivative thereof.

There is also provided in a further or alternative aspect of the invention use of a TNFα inhibitor as herein defined or a pharmaceutically acceptable derivative thereof for the manufacture of a medicament for the treatment of conditions associated with excessive or unregulated TNFα production.

It will be appreciated by those skilled in the art that reference herein to treatment extends to prophylaxis as well as the treatment of established conditions or symptoms.

It will be further appreciated that the amount of TNFα inhibitor of the invention required for use in treatment will vary not only with the route of administration but also with the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or veterinarian. In general however, a suitable dose will be in the range of from about 5.0 to 500 μg per kilogram of bodyweight per day,

for example, in the range 30 to 300µg/kg/day, preferably in the range 50 to 150µg/kg/day.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day.

While it may be possible that, for use in therapy, a TNFα inhibitor of the invention may be administered as the raw protein it is preferable to present the active protein as a pharmaceutical formulation.

The invention further provides a pharmaceutical formulation comprising a TNFα inhibitor as herein defined or a pharmaceutically acceptable derivative thereof together with one or more pharmaceutically acceptable carriers thereof and, optionally, other therapeutic and/or prophylactic ingredients. The carrier(s) must be 'acceptable' in the sense of being compatible with the ingredients of the formulation and not deleterious to the recipient thereof.

The inhibitors according to the invention may therefore be formulated for parenteral administration (e.g. by injection, for example bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, small volume infusions or in multi-dose containers with an added preservative. The compositions may take such forms as suspensions or solutions in aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the active ingredient may be in powder form obtained by aseptic isolation of sterile solid or by lyophilisation from solution, for constitution

with a suitable vehicle, e.g. sterile, pyrogen-free water, before use.

The TNFα inhibitor of the invention may also be used in combination with other therapeutic agents, for example, other cytokines or inhibitors thereof.

The invention thus provides, in a further aspect, a combination comprising a TNFα inhibitor as herein defined or a pharmaceutically acceptable derivative thereof together with another therapeutically active agent, for example, other cytokines or inhibitors thereof.

The proteins of the invention may be prepared by purification from natural sources and, where appropriate followed by chemical modification, or they may be prepared by conventional methods known in the art for the preparation of proteins, for example, by recombinant DNA techniques.

According to a further aspect of the present invention, there is provided a process for producing the tumour necrosis factor α inhibitor of the invention by purification from natural sources, particularly the urine of human febrile patients. Such purification, for example, comprises the steps of concentrating the crude urine of febrile human patients, precipitating crude TNFα INH from the urine and fractionating the TNFα INH from the other proteins of this precipitate by one or more of, for example, ion exchange column chromatography, gel filtration chromatography, hydrophobicity chromatography, immunoabsorption and affinity chromatography on immobilized TNFα.

The tumour necrosis factor α inhibitor of the invention is also obtainable from macrophage containing human tissue, for example,

lung lavages and extracts of human liver, from which it may be obtained by standard purification techniques such as those described above.

Natural and recombinant TNFα INH produced according to the

5   procedures described herein may be purified through a series of steps as listed above. After each of the purification steps, the presence and purity of the TNFα INH may be measured in an assay of cytotoxicity in the presence of actinomycin D (acti D) using a TNF-susceptible cell line L929, as described by G. Nedwin et al., J. Immunol., 135, loc.

10   cit.

In a preferred embodiment of the process the TNFα INH is initially isolated from untreated urine collected from febrile human patients (>38.5°C) devoid of urinary infections using a standard concentration technique, for example, ultrafiltration. A crude

15   fraction may then be precipitated from the crude urine using ammonium sulphate e.g. by addition of ammonium sulphate to a concentration of 80% (w/v) at 4°C with stirring. Preferably the ammonium sulphate may be added in a stepwise manner and material precipitated at lower concentrations e.g. at 40% (w/v) discarded. The ammonium sulphate may

20   be removed by dialysis and the resulting fraction purified to separate the TNFα INH from other proteins by a variety of chromatographic methods.

Thus, the TNFα INH concentrate may be purified by ion-exchange chromatography which separates proteins according to their differences

25   in electrical charge, which is a reflection of the acid/base properties of the proteins. Suitable materials for anion-exchange chromatography include aminoethylcellulose derivatives, for example,

quaternary-aminoethylcellulose (QAE-cellulose) or diethylaminoethyl-cellulose (DEAE-cellulose) which are widely commercially available. The anion-exchange column should be equilibrated prior to applying the concentrate using a suitable buffer, such as Tris-HCl, optionally

5   containing a chelating agent such as EDTA. Bound material may be eluted from the column using a salt solution (for example, 0.8M sodium chloride made up with the equilibration buffer).

The pooled active fractions from the anion-exchange chromatography. Suitable materials for cation-exchange chromatography

10   include derivatives of cellulose such as carboxymethyl (CM) cellulose or Sulphopropyl Sepharose (Pharmacia, Uppsala, Sweden). The column should be equilibrated with a suitable buffer, such as sodium acetate and bound material may be eluted with the equilibration buffer containing, for example, 0.5M sodium chloride.

15   The pooled active fractions are further purified by affinity chromatography on bound recombinant human TNFα (rhTNFα), coupled to a suitable matrix, for example, Mini-Leak Agarose (Kem En Tec, Biotechnology Corp., Denmark). The column should be buffered using, for instance, a phosphate buffer (e.g. 0.8M potassium phosphate pH

20   8.6). Active groups not bound to rhTNFα should be blocked using ethanolamine-HCl pH 8.5 buffer. The column should be equilibrated with a suitable buffer, for example, Tris-HCl optionally containing sodium chloride and the TNFα INH eluted with an acidic (pH 3.5) glycine buffer. The eluted fractions should immediately be balanced

25   to pH 7.0 by the addition of, for example, Tris base.

The active pooled fractions are preferably lyophilised prior to the final purification step of reverse-phase FPLC (fast protein liquid

chromatography). Prior to its application to the FPLC column, the
lyophilised TNFᵢ INH fraction should be buffered with an appropriate
buffer such as trifluoroacetic acid (TFA) (Fulka, Buchs, Switzerland),
heptafluorobutyric acid (HFBA) or acetic acid. Elution of the TNFᵢ

5   INH from the FPLC column may be carried out using conventional
techniques, for example, made up with a suitable buffer as previously
described, optionally containing an alcohol, for instance,
N-propanol.

The eluted fraction should immediately be buffered with, for

10 example, ammonium bicarbonate and lyophilised. The TNFᵢ INH is now in
a substantially homogenous form suitable for further assaying of
biological activity and for preparation in an appropriate form for
therapeutic use.

There is thus provided as a further or alternative aspect of the

15 invention a protein which selectively inhibits TNFα-mediated activity
which is substantially identical to that obtained in the above
process.

The ability to purify TNFᵢ INH of the present invention to
homogeneity has enabled the sequencing of the N-terminal portion of

20 this protein molecule. This sequence will assist in the cloning of a
gene coding for the TNFα INH thus enabling the production of large
quantities of TNFα INH in pure form for further biological
investigation and eventually for therapeutic testing and use.

Samples of the homogeneous TNFᵢ INH of the present invention can

25 be sequenced by conventional techniques, for example, using a
commercially available automated sequencer employing either ninhydrin
or gas phase detection. The first 17 residues of the amino terminal

portion of the human TNFα INH as herein defined comprises the following sequence :

Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-Cys-Asn-Ser-Ile

(identified using an automated sequencer, Model 477A from Applied Biosystems).

It is further believed that the next three amino acids provide a glycosylation site and that the sequence thus continues Asn-Ser-Thr-Lys.

It will be appreciated that one potential method of providing large quantities of TNFα INH in pure form is through recombinant DNA techniques which are well known in the art. However, the successful use of such techniques requires not only that the natural and recombinant TNFα INH or its activity be accurately measured, but also that both the natural and recombinant product be purifiable to homogeneity.

According to yet another aspect of the present invention, there is provided a process for producing a tumour necrosis factor inhibitor of the invention or a derivative thereof by the expression of a DNA sequence encoding for such an inhibitor in a suitable transformed host. Such a process involves the culturing of a host transformed with recombinant DNA molecules comprising DNA sequences encoding the inhibitor which have been inserted into a suitable vector.

Suitable eukaryotic and prokaryotic hosts may be, for example, strains of bacteria, yeasts, other fungi, and animal cells (including insect cells) and plant cells in tissue. Particularly preferred host

cells are yeast cells, E. coli cells and animal cells.

Expression of a protein having tumour necrosis factor inhibitor activity is achieved by culture of the transformed host cells in a suitable growth medium. Normally such a medium will contain a source of nitrogen such as ammonium sulphate, a source of carbon and energy such as glucose or glycerol, trace elements and factors essential to growth of the particular host cells. The precise culture conditions will be dependent upon the chosen host; thus, for example, in the case of E. coli submerged aerobic fermentation is preferred, preferably at about $37^{\circ}C$.

In addition, expression may be induced, for example, by the addition of an inducer or the use of inducing conditions for the promoter system being used in the expression vector.

Depending upon the host, the TNFα inhibitor may be produced as granular inclusion bodies which can be recovered, after cell lysis, by differential centrifugation; these can be solubilised by conventional methods and purified by the methods described herein for purification of urinary TNFα INH. Alternatively, the TNFα inhibitor may be in solution in the cytosol, secreted into the periplasmic space or conveniently secreted into the culture medium.

The host cells are transformed by recombinant DNA molecules which comprise a DNA sequence encoding for a TNFα inhibitor which has been inserted into an expression vector.

Such expression vectors may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV-40 and known bacterial plasmids, for example, "natural" plasmids such as ColEl, pSCIOI or pRSF2124 and

phage DNAs, or "artificial" plasmids (constructed in vitro) such as
pBR322, pMB9 or pAT153. Phage DNAs include, for example, the numerous
derivatives of phage lambda and other DNA phages, for example M13, and
other filamentous single-stranded DNA phages. Vectors useful in
yeasts include the 2u plasmid, and those useful in eukaryotic cells
such as animal cells include those containing SV-40 adenovirus and
retrovirus.

Such expression vectors may also be characterised by at least
one expression control sequence which may be operatively linked to the
TNF inhibitor DNA sequence such that it controls and regulates the
expression of the cloned DNA sequence. Examples of useful expression
control sequences include the lac, trp, tac and trc systems, major
operator and promoter regions of phage λ (such as the P_L promoter
under the control of the thermolabile ts cI857 repressor), the control
region of fd coat protein, the glycolytic promoters of yeast (e.g. the
promoter for 3-phosphoglycerate kinase), the promoters of yeast acid
phosphatase (e.g. Pho 5), the promoters of yeast α-mating factors, and
promoters derived from polyoma, adenovirus, retrovirus, and simian
virus.

In addition, such expression vectors may possess various sites
for insertion of the TNFα inhibitor DNA sequences of this invention.
These sites are characterised by the specific restriction endonuclease
which cleaves them. Such cleavage sites are well recognised by those
skilled in the art. The expression vector, and in particular the site
chosen therein for insertion of a selected DNA fragment and its
operative linking to an expression control sequence, is determined by
a variety of factors including the number of sites susceptible to a

given restriction enzyme, the size of the protein to be expressed, contamination or binding of the protein to be expressed by host cell proteins which may be difficult to remove during purification, the location of start/stop codons, and other factors recognised by those

5   skilled in the art. Thus the choice of a vector and insertion site for a DNA sequence is determined by a balance of these factors, not all selections being equally effective for a given case.

Likewise, not all host/vector combinations will function with equal efficiency in expressing the DNA sequences of this invention.

10 The selection is made, depending upon a variety of factors including compatability of the host and vector, ease of recovery of the desired protein, expression characteristics of the DNA sequences and the expression control sequences operatively linked to them, or any necessary post-expression modifications of the desired protein.

15 The DNA sequences of the invention which on expression code for proteins with TNFα inhibitor activity may be isolated by screening various DNA libraries for such DNA sequences using a series of DNA probes. The DNA probes may be prepared from the purified natural protein which is used as a source of amino acid sequence data. The

20 purified natural protein may be prepared, for example, from febrile human urine as described above. Degenerate DNA sequences coding for various portions and fragments of the amino acid sequence, e.g. in combination with Lathe probes, are used to design the DNA probes.

Thus, various DNA libraries are screened for DNA sequences

25 coding for the TNFα inhibitors of the invention. Such libraries include chromosomal gene banks and cDNA or DNA libraries prepared from cell lines or tissue that are demonstrated to produce TNFx

inhibitors, such as alveolar macrophages or liver tissue. Screening
may be by direct immune expression, for example in λgt11 or similar
systems, or, in the case where a TNFα INH producing cell is
identified, by identification of TNFα INH specific mRNA by direct

5   expression in Xenopus oocytes.

A variety of conventional cloning and selection techniques may
be used to locate and identify DNA sequences which encode on expression
in an appropriate eukaryotic or prokaryotic host for the TNFα
inhibitors of this invention. These selected DNA sequences may

10   themselves be used as probes to select other DNA sequences coding for
TNFα inhibitors or may be used in appropriate recombinant DNA
molecules to transform appropriate eukaryotic or prokaryotic hosts
for the production of TNFα INH encoded by them.

The invention includes within its scope single and double

15   stranded DNA sequences encoding for TNFα INHs, vectors containing such
sequences suitable for transformation of a host organism and host
cells transformed with such DNA sequences.

According to a further aspect of the present invention we
provide a protein with selective TNFα inhibitor activity produced by

20   expression of a host transformed with a DNA sequence encoding for such
a TNFα inhibitor protein. TNF inhibitors of the invention which are
prepared by the expression of a DNA sequence encoding such inhibitors
in a transformed host will thus be identical to the sequence of native
TNFα INH or contain one or more deletions, substitutions, insertions,

25   inversions or additions of allelic origin or otherwise, the resulting
sequence will have at least 80% and preferably 90% homology with the
sequence of native TNFα INH and retain essentially the same biological

properties. In particular, a TNF inhibitor of the invention may include an N-terminal methionine. Also, for example, the DNA sequence of the invention coding for TNFα INH may be fused in an expression vector to a portion of a DNA sequence coding for a eukaryotic or prokaryotic polypeptide to assist the expression of the TNFα INH encoding DNA sequence or aid secretion, maturation or purification of the TNFα INH from the host; the fused polypeptide may be removed intra-or extra-cellularly by known techniques or the INFα INH may be used together with the fused polypeptide.

The TNFα INHs produced by culturing of the eukaryotic and prokaryotic hosts transformed with DNA sequences encoding for TNFα INHs can then be employed, after purification, in the pharmaceutical compositions of this invention.

It will be appreciated that, when produced by animal cells, the TNFα INH of the invention will be a glycoprotein. Prokaryotic expression systems will, however, produce the protein in an unglycosylated state. In addition, the glycosylated protein may be substantially deglycosylated by techniques known in the art, for example, by the use of endoglycosidase enzymes.

The following non-limiting Examples illustrate the invention. All temperatures are in °C and all percentage concentrations are w\.

TNFα Inhibition Assay

The percentage of TNFα INH activity in the fractions described in the Examples was determined by assuming that the optical density (OD) values from murine L929 cells stimulated by actinomycin D (acti D) corresponded to 100% inhibition, whereas the OD from cells cultured

with actinomycin D and TNFα corresponded to a maximal cell mortality
of 0% TNFα inhibition. The TNFα used in the assay was recombinant
human TNFα (rhTNFα) produced in *E. coli* as described by A. Marmenout
et. al., "Molecular cloning and expression of human tumour necrosis
factor and comparison with mouse tumour necrosis factor", Eur. J.
Biochem., 152, p515 (1985). Thus the percentage of TNFα inhibition in
the assay of cytotoxicity was calculated according to formula (I)

Percentage of TNFα INH activity =

$$100 \times \left[\frac{(OD \text{ with acti } D + rhTNF\alpha + TNF\alpha \text{ INH}) - (OD \text{ with acti } D + rhTNF\alpha)}{(OD \text{ with acti } D) - (OD \text{ with acti } D + rhTNF\alpha)} \right] \quad (I)$$

Example 1

Purification of Urinary TNFα INH

a) Concentration of Protein from Human Urine

Human urine (15 litres) was freshly obtained from a pool of
five patients prior to any treatment. Two of the patients were
suffering from small-cell carcinoma, one from malignant histocytosis,
one from polymyocitis and one from sepsis. All were highly febrile
(>38.5°C) and devoid of urinary infections. The urine was
concentrated at 4° on an Amicon ultrafiltration hollow fibre
apparatus, with a molecular size cut-off of about 5 kDa.

b) Precipitation of Protein from Human Urine

The concentrated urine pool was saturated with solid ammonium sulphate by adding the sulphate slowly with constant stirring at 4° until an ammonium sulphate concentration of 40% was reached. The precipitate was removed by centrifugation, discarded, and the supernatant adjusted to 80% saturation with addition of further ammonium sulphate. A pellet was obtained by centrifugation which was resuspended in 150 ml of 20 mM sodium phosphate (pH 7.2) and 150 mM sodium chloride. The ammonium sulphate was removed by dialysis at 4° using 10 mM Tris-HCl pH 7.4, 2 mM EDTA and 5 mM benzamidine HCl.

c) Identification of TNFα INH Activity

The semi-purified fraction of Example 1(b) was tested in a cytoxicity assay with the TNF-susceptible cell-line L929 in the presence of actinomycin D. At a 1:20 dilution of the semi-purified fraction, total inhibition of the cytotoxic effect induced by rhTNFα was observed so that the OD_{570nm} value was identical to that measured in the presence of actinomycin D alone ($OD_{570nm} = 1.5$).

Furthermore, inhibitory activity was observed in dilutions of the fraction of up to 1:160 on cells ($OD_{570nm} = 0.83$) whereas the control value of rhTNFα at a final concentration of 0.2 ng/ml measured in the presence of actinomycin D was lower ($OD_{570nm} = 0.73$), so that 50% of inhibition was observed at a dilution of approximately 1:100 ($OD_{570nm} = 1.10$). The TNFα INH had no effect on cell viability when tested without actinomycin D.

d) Comparison of the Effect of TNFβ INH on TNF α and β Induced
Cytotoxicity

A cytotoxicity assay was conducted using the TNF-susceptible
cell-line L929 in the presence of actinomycin D using a range of
concentration of TNFα or TNFβ to induce the cytotoxic effect. The
semi-purified fraction from Example 1(b) was tested at 1:20, 1:50 and
1:90 dilutions. Control tests were performed in the absence of the
TNFα or TNFβ cytokine, and in the absence of inhibitor. The results
are shown in Table 1, below, and demonstrate that the inhibitor of the
invention does have some inhibitory effect on TNFβ mediated cytoxicity
ranging from approximately 50% down to 2% of the TNFα inhibition with
increasing TNFβ concentration.

TABLE 1

Final Concentration of TNF (α or β) (pg/ml) Added to Actinomycin-D Treated L929 cells	Form of Cytokine added to cells	Final Dilution of Sephacryl S-200 Inhibitory Fraction on L929 cells (OD$_{570nm}$)			
		None	1/20	1/50	1/90
0	0	>1.90	>1.90	>1.90	>1.90
10	α	ND	ND	ND	ND
	β	1.16	1.66	1.46	1.39
20	α	1.30	>1.90	1.72	1.68
	β	1.02	1.51	1.21	1.22
50	α	1.02	>1.90	1.72	1.68
	β	0.65	1.03	0.84	0.66
100	α	0.73	1.78	1.69	1.51
	β	0.37	0.71	0.59	0.52
250	α	0.38	1.70	1.70	1.33
	β	0.24	0.44	0.31	0.24
500	α	0.30	1.52	1.49	1.11
	β	0.17	0.30	0.26	0.18
1 250	α	0.19	1.09	1.10	0.76
	β	0.11	0.13	0.19	0.13
2 500	α	0.06	1.03	0.80	0.47
	β	ND	ND	ND	ND

Example 2

Gel Filtration of Urinary TNFα INH

The semi-purified TNFα INH of Example 1(b) was purified by gel filtration chromatography at 4° on a Sephacryl S-200 column (0.9 x 60 cm) (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM Tris-HCl buffer (pH7.4) containing 100 mM sodium chloride. A sample of the protein fraction (20 mg, 0.8 ml) was applied to the column and eluted with the same buffer at a flow-rate of 5.4 ml/hr. Fractions (1.35 ml) were collected and tested for TNFα INH activity. The TNFα INH activity eluted from the gel in a single peak. The inhibitory activity showed an apparent molecular weight of 40 to 60 kDa (see Figure 1).

Example 3

Chromatofocussing of Urinary TNFα INH

The semi-purified TNFα INH of Example 1(b) was chromatofocussed at 4° on a Mono-P pre-packed column (HR 5/20, 5 x 200 mm) (Pharmacia, Uppsala, Sweden) equilibrated in 25 mM Bis-Tris buffer adjusted to pH 7.1 with imidodiacetic acid (Fluka, Buchs, Switzerland). A sample of the protein fraction of Example 1(b) (30 mg) was applied to the column and eluted with a polybuffer 74/iminodiacetic acid at pH 4.0. Column fractions (1 ml) were tested at 1:10 dilution for their effect in the rhTNFα (0.2 ng/ml) cytotoxicity assay in the presence of actinomycin D (1ug/ml). The actual pH of each column fraction was determined with a pH meter, the bulk of the TNFα INH activity being contained in the eluted fractions

between pH 5.5 and 6.1 (see Figure 2). This is equivalent to the pI of the TNFa INH protein.

Example 4

Ion-Exchange Chromatography of Urinary TNFa INH

The semi-purified TNFa INH of Example 1(b) was purified by anion-exchange chromatography at 4° on a DEAE Sephadex column (2.6 x 20cm) (Pharmacia, Uppsala, Sweden) equilibrated in 10mM Tris-HCl buffer pH 8.0, containing 2mM EDTA. Bound material was eluted from the column with the equilibration buffer containing 0.8M sodium chloride. Fractions (8.0ml) were collected, tested for TNFa INH activity and the inhibitory fractions were pooled (160ml) and dialysed against 10mM sodium acetate buffer pH 5.0 (4 x 2 litres).

The TNFa INH was further purified by cation-exchange chromatography at 4° on a Sulphopropyl-Sephadex column (0.8 x 15 cm) (Pharmacia, Uppsala, Sweden) equilibrated in 10mM sodium acetate buffer pH 5.0. Bound material was eluted from the column with the equilibration buffer containing 0.5M sodium chloride. Fractions (7.5ml) were collected, tested for TNFa INH activity and the inhibitory fractions were pooled and concentrated 20-fold on an Amicon ultrafiltration apparatus with a molecular size cut-off of about 10 kDa.

Example 5

Gel Filtration of Urinary TNFa INH

The TNFa INH concentrate from Example 4 was purified by gel filtration chromatography at 4° on a Sephacryl S-200 column (2.6 x 100

cm) (Pharmacia, Uppsala, Sweden) equilibrated with 50mM Tris-HCl pH
7.4 buffer containing 100mM sodium chloride. A sample of the protein
fraction from Example 4 (200mg) was applied to the column and eluted
with the equilibration buffer at a flow-rate of 27ml/hour. Fractions
(9.0ml) were collected, tested for TNFα INH activity and the
inhibitory fractions were pooled. The column was calibrated with
dextran blue (DB), 2000 kDa; bovine serum albumin (BSA), 67 kDa;
ovalbumin (OA), 43 kDa; α-chymotrypsinogen-A (αCT), 25 kDa; and
ribonuclease A (RNase), 13.5 kDa, as shown in Figure 4.

Example 6

Affinity Chromatography of Urinary TNFα INH

A TNFα affinity column was prepared by coupling recombinant human
TNFα (1.0mg) to Mini Leak Agarose (Kem En Tec, Biotechnology Corp.,
Denmark) in 0.8M potassium phosphate buffer pH 8.6. The remaining
active groups were blocked by incubation in 0.1M ethanolamine-HCl
buffer pH 8.5. The gel was washed with 50mM Tris-HCl pH 7.4 buffer
containing 100mM sodium chloride (3 x 50ml). A sample of the TNFα INH
fractions from Example 5 (15ml) was applied to the column and eluted
with a 0.2M glycine-HCl pH 3.5 buffer. Fractions (1.0ml) were
collected, immediately adjusted to pH 7.0 by addition of 1M Tris (5 to
40μl) and tested for TNFα INH activity.

Example 7

Reverse Phase FPLC Chromatography of Urinary TNFα INH

The TNFα INH fractions from Example 6 were lyophilised, dissolved
in 0.1% trifluoroacetic acid (2.0ml) and loaded onto a ProRPC

reverse-phase FPLC column (5 x 20 cm) (Pharmacia, Uppsala, Sweden)
equilibrated in 0.1% trifluoroacetic acid. Bound material was eluted
with a 0 to 100% acetonitrile gradient in 0.1% trifluoroacetic acid at
a flow rate of 0.3ml/minute. To each fraction (0.75ml) 0.5M ammonium
bicarbonate (10μl) was added and the eluted material was lyophilised.

The reverse-phase FPLC chromatography revealed one major peak
corresponding to TNFα INH activity. The lyophilised fractions
containing this activity were dissolved in 10mM Tris-HCl pH 7.4 buffer
containing 2mM EDTA and analysed by sodium dodecyl sulphate
polyacrylamide gel electrophoresis (SDS PAGE) using the method
described by U. Laemmli et al., Nature, 277, p 680 (1970). The TNFα
INH was found to elute with a molecular weight of 33 kDa (see Figure
4). Samples run under non-reducing conditions were tested for TNFα
INH activity at 1:10 dilution on L929 cells in the presence of 0.15
mg/ml of rhTNFα. The activity directed against rhTNFα migrated with
an apparent molecular weight identical to the 33 kDa band on the gel
run under reducing conditions.

Example 8

Protein Sequencing of Urinary TNFα INH

The TNFα INH fraction isolated from the reverse-phase FPLC
chromatography was concentrated in vacuo and spotted onto a
conditioned sequencer filter. The protein was analysed with an
Applied Biosystems Model 477A protein sequencer. Fractions from the
sequencing cycles were evaporated to dryness and resuspended in
N,N-diisopropylethylamine acetate and acetonitrile prior to injection
into an HPLC column for residue identification.

The first 17 amino acid residues of the N-terminal were identified and have the sequence : Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-Cys-Asn-Ser-Ile. It is further believed that the next three amino acids provide a glycosylation site and that the sequence thus continues Asn-Ser-Thr-Lys. This sequence is not significantly homogeneous to any protein sequence contained in the NBRF Protein Sequence database (November 1988).

Example 9

Demonstration that TNFα INH is a Protein

a) Time and Temperature Dependency

The Sephacryl S-200 purified TNFα INH of Example 2, obtained by bulking the tubes of the active fractions, was heated at 56°, 75° and 95°. The TNFα INH activity was measured after 10, 20 and 60 minutes and, by comparison with untreated samples, the percentage of TNFα INH activity was calculated according to formula (I). The results shown in Table 2 below demonstrate that the TNFα INH activity decreases in a time- and temperature-dependent manner.

Heat inactivation		
Temperature (^{o}C)	Time (min)	Percentage of TNFα INH activity
56	10	100
	20	100
	60	93
75	10	60
	20	26
	60	15
95	10	27
	20	10
	60	13

b) Susceptibility to Trypsin Digestion

Trypsin (500 μg) (Sigma, St. Louis, MO) in 0.2M Tris-HCl buffer
(pH 8.0) containing 1mM calcium chloride was added to the pooled
fractions of Sephacryl S-200 purified urinary TNFα INH of Example 2
and incubated at 37°C for 4 hours. Another measure of trypsin (500 μg)
was added and digestion continued for a further 20 hours, at which
time the reaction was terminated by addition of soybean trypsin
inhibitor (2mg) (Sigma, St. Louis, MO). The percentage of TNFα INH
inhibitory activity of the trypsin digest and the control was
determined at a 1:20 final dilution of the pool of bulked fractions on
L929 cells stimulated by rhTNFα in the presence of actinomycin D,
according to formula (I). The results are shown in Table 3 below.

Trypsin inactivation		
Conditions	Pecentage of TNFx INH activity	CD 570nm
Buffer alone	0	0.71
Trypsin + soybean trypsin inhibitor in buffer	0	0.70
Partially purified Sephadex S-200 urine	61	1.46
Partially purified Sephadex S-200 urine digested by trypsin	23	1.03

c) Treatment with Urea

The Sephacryl S-200 purified TNFα INH of Example 2 was
adjusted to 2M urea and extensively dialysed at 4°C against phosphate
buffered saline (PBS) containing 2M urea.   Dialysis against PBS was
repeated prior to the bioassay.   TNFα INH activity was found to be
unaffected which indicates that inhibitory activity is not mediated by
a molecule of low molecular weight bound to a protein.

Example 10

Demonstration of Competitive Inhibition

The Sephacryl S-200 purified TNFα INH of Example 2 was tested
at a 1:10 dilution against increasing amounts of rhTNFα on L929 cells.
An inverse correlation between the amount of rhTNFα present in the
assay and the degree of inhibition was observed (see Figure 3).   Thus,
the inhibitory activity is competitively overcome by increasing
concentrations of rhTNFα.

Example 11

Inhibition of TNFα-Mediated PGE$_2$ Production by Dermal Fibroblasts

Human dermal fibroblasts were seeded at a concentration of 2.0 x 10^4 cells/well and cultured for 48 hours. Cells were then stimulated with DMEM buffer supplemented with 10% FCS as a control. Cells were also stimulated with rhTNFα at concentrations ranging from 0.5 to 5mg/ml, and the effect of the TNFα INH from Example 5 was studied at three dilutions (1:20, 1:50 and 1:80) in the above buffer. After 72 hours of incubation, PGE$_2$ production was measured in the supernatants by radioimmunoassay using a PGE$_2$ antiserum [see J M Dayer et al., J. Clin. Invest., 67, p1386 (1979)].

The results are shown in Table 4, below, and demonstrate that the ability of rhTNFα to stimulate PGE$_2$ production by dermal fibroblasts was inhibited by the addition of TNFα INH at all three dilutions. At 1:80 dilution of TNFα INH the inhibitory activity was partially overcome by increasing rhTNFα concentrations.

| Concentration of rhTNF on human fibroblasts (pg/ml) | PGE$_2$ production by human dermal fibroblasts (ng/ml) | | | |
| | Dilution of TNF INH on fibroblasts | | | |
	none	1:80	1:50	1:20
0	50.6 ± 7.4	86.8 ± 5.6	103.0 ± 8.9	111.0 ± 9.4
500	160.0 ± 14.1	126.0 ± 9.3	115.9 ± 6.6	113.9 ± 7.1
2,000	331.7 ± 28.4	217.2 ± 10.7	156.7 ± 10.7	115.3 ± 21.3
5,000	381.7 ± 19.6	257.2 ± 13.7	253.1 ± 21.2	221.6 ± 16.0

Three different experiments were carried out with the same strain of fibroblasts. Buffer or TNFα INH was incubated at various dilutions in the presence or absence of various concentrations of rhTNFα. PGE$_2$ production by cultured human dermal fibroblasts was measured after three days. Values represent triplicate means of the three cultures ± SEM (N=3).

Example 12

rhTNFα Binding Inhibition Assay

Recombinant human TNFα was iodinated by using the iodogen method of
Fraker and Soeck jr., Biochem. Biophys. Res. Comm., 80, p849 (1978).
The specific activity of $[^{125}I]$-TNFα was 2.2×10^4 cpm/ng and produced a
single band with a molecular weight of 17 kDa when analysed by SDS
PAGE. The human macrophage cell line U937 in aliquots of 10^6 cells
was cultured at 4^0 for 2 hours in a culture medium (200µl) comprising
RPMI 1640 (Gibco, Paisley, Scotland) supplemented with streptomycin
(100µg/ml), penicillin (100U/ml), 1.0% glutamine and 10% foetal calf
serum, and additionally containing 0.04% sodium azide and 0.5ng
$[^{125}I]$-TNFα. Binding inhibition was performed by the addition of
various dilutions of TNF INH (1:20, 1:200 and 1:2000).

Non-specific binding was measured in the presence of a 100-fold
excess of unlabelled rhTNFα, and free radioactivity was separated
from the bound $[^{125}I]$-TNFα by centrifugation through an oil mixture as
described by Robb et al., J. Exp. Med., 154, p1455 (1981). Cell
bound $[^{125}I]$-TNF was measured in a gamma counter (LKB, Bromma,
Sweden), and the percentage of binding inhibition was determined
according to formula (II)

Percentage of binding inhibition =

$$100 \times \left[1 - \frac{\text{cpm with TNF INH} - \text{cpm non-specific binding}}{\text{cpm of total binding} - \text{cpm non-specific binding}} \right] \quad (II)$$

Example 13

Effect of TNFα INH on [125I]-TNFα Binding to U937 Cells

U937 cells were preincubated for 1 hour at 20^c in the culture medium of Example 12, in the presence of either [125I]-TNFα alone or [125I]-TNFα with a 100-fold excess of unlabelled rhTNFα. The U937 cells were then washed with phosphate buffered saline (3 x 50ml) at 4^0. The cells incubated in [125I]-TNFα alone were divided into four batches and incubated with TNFα INH from Example 5 (1:20, 1:200 and 1:2000 dilutions) and with buffer alone, respectively.

The specific binding of [125I]-TNFα to U937 cells was found to be inhibited at 4^0 by 100%, 80% and 35% by the three dilutions of TNFα INH, 1:20, 1:200 and 1:2000, respectively (see Figure 5). The control batch which lacked TNFα INH showed no inhibitory activity. The binding inhibition of the two weaker dilutions was found to be increased to 90% and 60% when [125I]-TNFα was preincubated with TNFα INH at dilutions 1:200 and 1:2000, respectively, prior to cell addition.

The experiment was repeated using the cells preincubated in the presence of [125I]-TNFα and a 100-fold excess of unlabelled TNFα so that the percentage of binding inhibition could be corrected for non-specific binding.

Example 14

Dissociation of a Pre-Formed TNFα:U937 Complex

U937 cells were preincubated for 1 hour in the presence of [125I]-TNFα as described in Example 12. The cells were washed and incubated at either 4^0 or 37^0 in the presence or absence of TNFα INH

from Example 5. Cell-surface bound [¹²⁵I]-TNFα was found to dissociate faster in the presence of TNFα INH than in its absence and this was found to occur in a time- and temperature-dependent manner (see Figure 6).

Example 15

Demonstration that TNFα INH is not Proteolytic for rhTNFα

[¹²⁵I]-TNFα was incubated at 20° for 1 hour in the presence of 3 different dilutions of TNFα INH (1:20, 1:200 and 1:2000) and in the presence of buffer alone. When analysed by SDS PAGE and autoradiography the [¹²⁵I]-TNFα was found to migrate as a single band both in the absence and presence of TNFα INH showing the inhibitor to have no proteolytic effect.

Example 16

Effect of TNFα INH on IL-1 Receptor Binding Activity

The activity of TNFα INH from Example 5 was tested in the IL-1/LAF (lymphocyte activating factor) assay when induced by IL-1α or IL-1β [this assay is described by P. Seckinger et al., J. Immunol., 139, p1541 (1987) for an IL-1 inhibitor protein]. A dose response of [³H]-thymidine incorporation (corresponding to thymocyte proliferation) was observed in up to 200pg/ml concentrations of both IL-1α and IL-1β. Addition of TNFα INH at levels observed to inhibit rhTNFα did not have any significant effect on the IL-1-induced thymocyte proliferation, proving inhibition to be specific for TNFα only.

The results obtained are illustrated by reference to the accompanying drawings, in which:-

Figure 1 - shows the urinary TNFα INH activity profile of Sephacryl S-200 gel filtration. Column fractions (1ml) were tested at 1:10 dilution for effect in the rhTNFα (1.0ng/ml) cytotoxicity assay in the presence of actinomycin D (1.0μg/ml) (o——o). The line (——) represents OD_{280nm} of the fractions. Bars represent cell lysis measured by dye uptake in response to actinomycin D (☐) and to actinomycin D plus hrTNFα (☒) without urine. The molecular weight markers are dextran blue (DB), bovine serum albumin (BSA), ovalbumin (OA), α-chymotrypsinogen (αCT), ribonuclease A (RNase) and phenol red (θ-red).

Figure 2 - shows the urinary TNFα INH activity profile of chromatofocussing on a Mono-P column. Column fractions (1ml) were tested at 1:10 dilution for effect in the rhTNFα (0.2ng/ml) cytotoxicity assay in the presence of actinomycin D (1.0 μg/ml) (o——o). The line (——) represents OD_{280nm} of the fractions, and (-----) represents their pH. The bars are as described for Figure 1.

Figure 3 - shows the reversibility of TNFα INH activity. Open circles (o——o) represent OD_{570nm} measured in the presence of actinomycin D, rhTNFα with TNFα INH; closed circles (●——●) represent OD_{570nm} measured in the presence of actinomycin and rhTNFα only and the bar (☒) represents OD_{570nm} in the presence of actinomycin D (1.0μg/ml) alone.

Figure 4 - shows the elution profile of Sepacryl S-200 gel filtration with purified TNFα INH from Example 5. Column fractions (9ml) were sterilized and tested at 1:50 dilution against rhTNFα (1.0mg/ml) in the presence of actinomycin D (1.0ug/ml) in the presence of actinomycin D (1.0ug/ml) in the L929 cytotoxicity assay (o——o). The line (————) represents OD_{280} nm of the fractions. The bars are as defined for Figure 1.

Figure 5 - shows the SDS PAGE analysis of purified TNFα INH of Example 7. SDS PAGE was performed as described by U. Laemmli et al., Nature, 277, loc cit. Samples were loaded onto 15% polyacrylamide gel with a 3% stacking gel and gels were silver-stained as described by C. Merril et al., Proc. Natl. Acad. Sci. USA, 76, p4335 (1979). Samples run under non-reducing conditions were tested for biological activity by cutting 2mm slices from the gel and eluting the proteins by overnight incubation in 10mM Tris-HCl pH 7.4 containing 2mM EDTA (total volume 200ul). Fractions were tested at 1:10 dilution on L929 cells in the presence of rhTNFα (0.15ng/ml).

Figure 6 - shows the effect of TNFα INH on $[^{125}I]$-TNFα binding to U937 cells. TNFα INH was incubated at three different dilutions in the presence of $[^{125}I]$-TNFα with the U937 cell line as described in Example 13. Open squares (□——□) represent incubation in the presence of TNFα INH and the open triangles (△——△) represent the control. Closed symbols refer to a 30 minute pre-incubation of TNFα INH at 20^0 with $[^{125}I]$-TNFα in the culture medium prior to cell addition.

Figure 7 - shows the dissociation of a pre-formed TNFα : U937
complex. U937 cells were pre-incubated with $[^{125}I]$-TNFα, washed and
incubated with TNFα INH as described in Example 14, at either 4°
(□——□) or 37° (■——■). At the time indicated, cell associated
radioactivity was measured and percentage specific binding determined.
On the graph, the value obtained from the control without the
inhibitor has been substracted from the values obtained at the two
temperatures, thus, 100% corresponds to the value obtained without the
addition of TNFα INH.

CLAIMS

1.   A protein which inhibits tumour necrosis factor (TNF) α-mediated activity but does not block other proteins which have in common with TNF certain but not all of the biological activities of TNF.

2.   A protein which selectively inhibits tumour necrosis factor α-mediated activity and having one or more of the following characteristics :

(a) a molecular weight in the range 40 to 60 kDa, determined by molecular sieve chromatography;

(b) an iso-electric point (pI) in the range 5.5 to 6.1, determined by chromatofocussing;

(c) inhibition of the standard TNF assay of differential cytotoxicity for murine L929 cells treated with actinomycin D;

(d) inhibition of TNF-induced PGE_2 release from human fibroblasts and synovial cells;

(e) the inhibitor interferes with the binding of TNFα to U937 cells (a monocytic tumour line) as evidenced by inhibition of binding of radiolabelled TNFα (^{125}I-TNFα);

(f) the dissociation of a pre-formed TNFα : U937 cell complex is promoted by the inhibitor in a temperature dependent manner;

(g) the inhibitor does not degrade TNF by proteolytic cleavage;

(h) the inhibitor does not inhibit IL-1 receptor-binding activity e.g. the binding of radiolabelled IL-1 (^{125}I-IL-1α) to the murine thymoma subline EL4-6.1.

3. A protein which selectively inhibits tumour necrosis factor α-mediated activity and having one or more of the following characteristics :

(a) a molecular weight of about 33 kDa, determined by SDS PAGE;

(b) an iso-electric point (pI) in the range 5.5 to 6.1, determined by chromatofocussing;

(c) inhibition of the standard TNF assay of differential cytotoxicity for murine L929 cells treated with actinomycin D;

(d) inhibition of TNF-induced PGE_2 release from human fibroblasts and synovial cells;

(e) the inhibitor interferes with the binding of TNFα to U937 cells (a monocytic tumour line) as evidenced by inhibition of binding of radiolabelled TNFα (^{125}I-TNFα);

(f) the dissociation of a pre-formed TNFα : U937 cell complex is promoted by the inhibitor in a temperature dependent manner;

(g) the inhibitor does not degrade TNF by proteolytic cleavage;

(h) the inhibitor does not inhibit IL-1 receptor-binding activity e.g. the binding of radiolabelled IL-1 (^{125}I-IL-1α) to the murine thymoma subline EL4-6.1.

4. A protein as claimed in either of claims 2 or 3 having the properties (a) and (b) together with one or more of the properties (c) to (h).

5.   A protein as claimed in either of claims 2 or 3 having all of the properties (a) to (h).

6.   A protein according to any one of claims 1 to 5 which corresponds to a naturally occurring TNFα inhibitor.

7.   A protein according to any one of claims 1 to 6 having an amino terminal amino acid sequence as follows :

   Asp-Ser-Val-Cys-Pro-Gln-Lys-Tyr-Ile-His-Pro-Gln-Cys-Asn-Ser-Ile.

8.   A protein according to claim 7 having an amino terminal amino acid sequence as follows :

Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-Cys-Asn-Ser-Ile-Asn-Ser-Thr-Lys.

9.   A protein according to any one of claims 1 to 8 and in which the amino acid sequence contains one or more deletions, substitutions, insertions, inversions or additions of allelic origin or otherwise, the resulting sequence having at least 80% homology with the parent protein and retaining essentially the same biological properties as the parent protein.

10. A protein according to claim 9 having at least 90% homology with the parent protein.

11. A protein according to any one of claims 1 to 10 in a substantially homogeneous form.

12. A protein according to any one of claims 1 to 11 which is a recombinant protein.

13. A protein according to any one of claims 1 to 12 which is a glycosylated protein.

14. A protein according to any one of claims 1 to 12 which is in a substantially unglycosylated state.

15. An exogeneous DNA comprising a nucleotide sequence coding for a protein as defined in any one of claims 1 to 11.

16. A cDNA comprising a nucleotide sequence coding for a protein according to any one of claims 1 to 11.

17. A recombinant expression vector comprising DNA according to either of claims 15 or 16.

18. A host cell transformed with an expression vector according to claim 17.

19. A method of producing a TNFα INH protein which comprises culturing a cell according to claim 18 and isolating the TNFα INH protein.

20. A recombinant protein produced according to the method of claim 19.

21. A method for the preparation of a TNFα INH protein comprising the steps of :

(a) concentration of urine from febrile patients;

(b) ammonium sulphate precipitation;

(c) anion-exchange chomatography;

(d) cation-exchange chromatography;

(e) gel filtration;

(f) affinity chromatography; and

(g) reverse phase FPLC.

22. A protein characterised in that it is substantially identical to the protein obtained by the method of claim 21.

23. A pharmaceutical formulation comprising a TNFα inhibitor as defined in any one of claims 1 to 14 or claim 22 or a pharmaceutically acceptable derivative thereof and a pharmaceutically acceptable carrier therefor.

24. A protein as defined in any one of claims 1 to 14 or claim 22 for use in therapy.

25. A pharmaceutical formulation for use in the manufacture of a medicament for the treatment of conditions associated with excessive or unregulated TNFα production, wherein said formulation comprises a TNFα inhibitor as defined in any one of claims 1 to 14 or claim 22 or or a pharmaceutically acceptable derivative thereof.